

RESEARCH ARTICLE

# The oxime pro-2-PAM provides minimal protection against the CNS effects of the nerve agents sarin, cyclosarin, and VX in guinea pigs

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## Abstract

This study examined whether pro-2-PAM, a pro-drug dihydropyridine derivative of the oxime 2-pralidoxime (2-PAM) that can penetrate the brain, could prevent or reverse the central toxic effects of three nerve agents; sarin, cyclosarin, and VX. The first experiment tested whether pro-2-PAM could reactivate guinea pig cholinesterase (ChE) *in vivo* in central and peripheral tissues inhibited by these nerve agents. Pro-2-PAM produced a dose-dependent reactivation of sarin- or VX-inhibited ChE in both peripheral and brain tissues, but with substantially greater reactivation in peripheral tissues compared to brain. Pro-2-PAM produced 9–25% reactivation of cyclosarin-inhibited ChE in blood, heart, and spinal cord, but no reactivation in brain or muscle tissues. In a second experiment, the ability of pro-2-PAM to block or terminate nerve agent-induced electroencephalographic seizure activity was evaluated. Pro-2-PAM was able to block sarin- or VX-induced seizures (16–33%) over a dose range of 24–32 mg/kg, but was ineffective against cyclosarin-induced seizures. Animals that were protected from seizures showed significantly less weight loss and greater behavioral function 24 h after exposure than those animals that were not protected. Additionally, brains were free from neuropathology when pro-2-PAM prevented seizures. In summary, pro-2-PAM provided modest reactivation of sarin- and VX-inhibited ChE in the brain and periphery, which was reflected by a limited ability to block or terminate seizures elicited by these agents. Pro-2-PAM was able to reactivate blood, heart, and spinal cord ChE inhibited by cyclosarin, but was not effective against cyclosarin-induced seizures.

**Keywords:** *Acetylcholinesterase; brain; central nervous system; cholinesterase inhibitors; cholinesterase reactivation; guinea pig; nerve agents; organophosphorus compounds; oximes; pralidoxime; pro-2-PAM; sarin; cyclosarin; VX.*

**Abbreviations:** ACh, acetylcholine; AChE, acetylcholinesterase; BBB, blood–brain barrier; BCA, bicinechoninic acid; ChE, cholinesterase; CNS, central nervous system; CS, conditioned stimulus; DFP, diisopropylfluorophosphate or fluostigmine or dyflos; EEG, electroencephalogram; HI-6, 1-(4-carbamoylpyridino)methoxymethyl-2-(hydroxyiminomethyl) pyridinium dichloride; HPLC, high pressure liquid chromatography; im, intramuscular; iv, intravenous; ITI, intertrial interval; LD<sub>50</sub>, median lethal dose; MMB-4, methoxime or 1,1'-methylene-bis[4-(hydroxyimino) methyl] pyridinium dichloride; OP, organophosphorus compound; 2-PAM, pralidoxime or pyridine-2-aldoxime methylchloride; PBF, phosphate buffered formalin; pro-2-PAM, 1-methyl-1,6-dihydropyridine-2-carbaldoxime; RBC, red blood cell; sc, subcutaneous; VX, o-ethyl S-(2-(diisopropylamino)ethyl)methylphosphonothioate; WB, whole blood

## Introduction

Chemical warfare nerve agents and certain pesticides are organophosphorus compounds (OPs) that potently inhibit cholinesterases (ChEs), in particular acetylcholinesterase (AChE), an enzyme that hydrolyzes the cholinergic neurotransmitter acetylcholine (ACh) in the peripheral and central nervous system (CNS) (Taylor 2001). The inhibition of AChE

leads to a build-up of ACh in the nervous system, resulting in a variety of signs and symptoms such as hypersecretion, bronchoconstriction, miosis, muscular twitching, mental confusion, convulsive seizures, respiratory distress, flaccid paralysis, and death (McDonough and Shih 1997; Ecobichon 2001; Taylor 2001; Eddleston et al. 2004b). In the US military, nerve agent poisoning is treated by administration of atropine

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sulfate, a drug used to antagonize muscarinic receptor binding by excess ACh, and 2-PAM (pralidoxime; pyridine-2-aldoxime methylchloride), an oxime drug used to reactivate inhibited AChE. Diazepam, a benzodiazepine drug, is also used in an effort to prevent brain seizures and motor convulsions caused by exposure to nerve agents (Moore et al. 1995; Lallement et al. 1997; Taylor 2001; Eddleston et al. 2004a).

The brain is a major target for the toxic effects of nerve agents. Inhibition of AChE in the brain results in seizures and neuropathology and contributes to the incapacitating behavioral and lethal effects of these OP agents (McDonough and Shih 1997; Shih et al. 2003; 2007). Protecting and/or restoring AChE activity in the brain is a major goal in the treatment of nerve agent intoxication. A potential way of restoring brain AChE is by the use of a centrally active oxime. However, treatment of nerve agent-inhibited brain AChE is complicated in that all current clinically used oximes are quaternary compounds with limited ability to cross the blood-brain barrier (BBB). Experimental evidence using an *in vivo* microdialysis technique indicates that brain penetration by 2-PAM in male Wistar rats is ~ 10%, but it is uncertain that this amount is effective at reactivating nerve agent-inhibited AChE (Sakurada et al. 2003). Nevertheless, studies have indicated that when laboratory animals are treated with an oxime that can cross the BBB, reactivation of inhibited CNS AChE does occur following OP nerve agent exposure (Rutland 1958; Shek et al. 1976b; Rump et al. 1978; Clement 1979; Boskovic et al. 1980; Heffron and Hobbiger 1980; Kenley et al. 1982; Talbot et al. 1986; Shih et al. 2009b; 2010a; b).

Bodor et al. (1976) were the first to synthesize a pro-drug dihydropyridine derivative of 2-PAM, pro-2-PAM, which is oxidized to 2-PAM in the peripheral tissues and CNS, in an attempt to get an oxime across the BBB. Subsequent studies in beagle dogs found that the conversion of pro-2-PAM to 2-PAM had a conversion half-life ( $t_{1/2}$ ) of 1.04 min and a biological  $t_{1/2}$  of 168 min, 60 min longer than that of native 2-PAM (Shek et al. 1976a). In a study using mice,  $^{14}\text{C}$ -labeled 2-PAM and pro-2-PAM were administered intravenously (i.v.) and the percentage of label determined in whole brain 15 min after injection by scintillation counting. It was found that 1.5% of the administered pro-2-PAM dose was detectable in the brain, 13-times higher than the equivalent dose of 2-PAM (~ 0.12%), and that pro-2-PAM was able to reactivate AChE inhibited by  $0.5 \times \text{LD}_{50}$  DFP (dyflos, fluostigmine; diisopropyl fluorophosphate) (Shek et al. 1976b). This amount of 2-PAM present in the brain (~ 0.12%) of mice in the Shek et al. (1976b) study differs significantly from the 10% reported by Sakurada et al. (2003) in rats, but this discrepancy most likely is due to differences in detection methods—measurement of whole brain total radioactivity (Shek et al.) vs HPLC analysis of striatal microdialysis samples (Sakurada et al.) and sampling times—cumulative radioactivity in brain 15 min after injection (Shek et al.) vs 60 min cumulative dialysate fraction from striatum after injection (Sakurada et al.). Further studies have evaluated pro-2-PAM for its efficacy against OP intoxication, with the results of these studies being ambiguous. Rump et al. (1978) showed that administration of pro-2-PAM

significantly increased the convulsive dose of DFP by 1.83-times in mice. Clement (1979) found that while administration of pro-2-PAM resulted in higher levels of mouse brain AChE activity, there was no correlation between brain AChE activity and survival, and suggested that pro-2-PAM did not offer a significant improvement over 2-PAM in animals challenged with either DFP or the nerve agent sarin. In another study, Talbot et al. (1986) found that pre-treatment with pro-2-PAM provided better protection than 2-PAM against lethal sarin intoxication in rats, but not against the nerve agents VX and soman. Most recently, Gordon et al. (2008) and DeMar et al. (2010) reported that pro-2-PAM protected brain AChE of guinea pigs challenged with DFP and also protected them from DFP-induced seizures, hypothermia, and bradycardia. Given these diverse results, the purpose of the present study was to systematically evaluate the capacity of pro-2-PAM to protect against the CNS effects of three OP nerve agents, sarin, cyclosarin, and VX, in guinea pigs.

## Materials and methods

### Subjects

Male Hartley guinea pigs (CrI:(HA) BR COBS) weighing 250–350 g were purchased from Charles River Labs (Kingston, NY). They were housed in individual cages in temperature ( $21 \pm 2^\circ\text{C}$ ) and humidity ( $50 \pm 10\%$ ) controlled quarters that were maintained on a 12-h light-dark schedule (with lights on at 06:00 h). Guinea pig diet #7006 (Harlan Teklad, Madison, WI) and filtered tap water were freely available whenever the animals were in home cages.

### Materials

Pro-2-PAM (1-methyl-1,6-dihydropyridine-2-carbaldoxime) was obtained from Southwestern Research Institute (San Antonio, TX). MMB-4 (methoxime; 1,1'-methylene-bis[4-(hydroxyimino)methyl]pyridinium dichloride) was obtained from the depository at Walter Reed Army Institute of Research (Silver Spring, MD). Acetylthiocholine iodide, atropine methyl nitrate, and atropine sulfate were purchased from Sigma-Aldrich (St. Louis, MO). Bicinchoninic acid (BCA) Protein Assay Reagent A (sodium carbonate, sodium bicarbonate, BCA<sup>+</sup> detection reagent, and sodium tartrate in 0.1 N sodium hydroxide) and BCA Protein Assay Reagent B (4% cupric sulfate) were purchased from Pierce Biotechnology, Inc. (Rockford, IL). Sarin (isopropyl methylphosphonofluoridate), cyclosarin (cyclohexyl methylphosphonofluoridate), and VX (0-ethyl S-(2-(diisopropylamino)ethyl) methylphosphonothioate) were obtained from the US Army Edgewood Chemical Biological Center (Aberdeen Proving Ground, MD). Nerve agents were diluted in ice-cold saline prior to subcutaneous (s.c.) injection. Pro-2-PAM was prepared freshly in citrate buffer (pH = 3) or saline in concentrations to deliver 0.5 ml/kg, intramuscularly (i.m.). In the reactivation experiment, atropine methyl nitrate and oxime were prepared in saline individually for i.m. injection. In the seizure experiment, atropine sulfate was prepared in saline. Injection volumes were 0.5 ml/kg for nerve agent and all treatment drugs.

### **Treatment dose rationale**

The doses of atropine sulfate and pro-2-PAM used in this study were human relevant doses of these drugs. The atropine sulfate dose (0.5 mg/kg) was the human equivalent of three 2-mg atropine autoinjectors (Nerve Agent Antidote MARK I Kit®) based on inter-species body surface area scaling formulas recommended by the Food and Drug Administration (FDA 2005). A 2-PAM dose of 25.0 mg/kg is the human equivalent of three 600-mg 2-PAM autoinjectors (Nerve Agent Antidote MARK I Kit®) on a mg/kg basis in a 70-kg individual (Hurst et al. 2007), and this dose was used to calculate a pro-2-PAM dose equivalent. In the reactivation and seizure control experiments, a dose range for pro-2-PAM (12.5–43.0 mg/kg) was conducted. MMB-4 (26.0 mg/kg; 58.0 µmol/kg dose, equivalent to three autoinjector doses based on HI-6 (Claire et al. 2000)) was administered i.m. The dose of this oxime was chosen because it results in high therapeutic effectiveness without any toxic effects in guinea pigs (Shih et al. 2009a).

### **Reactivation experiment**

Prior to the experiment, individual control blood samples (0.25–0.50 ml) were drawn using the toenail clip method (Vallejo-Freire 1951), collected into microfuge tubes containing 50 µl of heparin sodium (15 units/ml), and separated into whole blood (WB) and red blood cell (RBC) samples for ChE activity analysis. A 20-µl WB sample was collected and stored at –70°C until use. The remainder of the collected blood was used to prepare the RBC sample. The RBC sample was prepared by centrifugation (16,000 × g, 5 min) from the collected whole blood. A 10-µl sample of the packed RBC was mixed gently into 490 µl of 0.1% Triton-X 100 buffer and stored at –70°C until use. On the day of the study, guinea pigs were pre-treated 15 min prior to nerve agent exposure with the peripherally acting muscarinic receptor blocker atropine methyl nitrate (AMN; 1.0 mg/kg, i.m.), which does not affect ChE activity, to minimize peripheral toxic effects. AMN was selected for this experiment because it does not interfere with pharmacological events within the brain. Animals were then injected s.c. with either saline (0.5 ml/kg) or a 1.0 × LD<sub>50</sub> dose of sarin (42.0 µg/kg), cyclosarin (57.0 µg/kg), or VX (8.0 µg/kg). Fifteen minutes after nerve agent injection, when the inhibition of ChE activity by these nerve agents reached maximum (Shih et al. 2005), saline (0.5 ml/kg) or pro-2-PAM (12.5, 17.7, 25.0, or 35.3 mg/kg) was given i.m. Control animals received s.c. saline (no nerve agent) and i.m. saline (no oxime). Sixty minutes after saline or nerve agent administration (a time of significant reactivation of ChE by oximes), the animals were deeply anesthetized with isoflurane and euthanized by decapitation. Blood was collected and divided into WB and RBC samples. Brain regions (brainstem, cerebellum, cortex, hippocampus, midbrain, spinal cord, and striatum) and peripheral tissue (diaphragm, heart, and skeletal muscle) were dissected. Samples were processed for ChE activity and protein concentrations according to the methods previously described (Shih et al. 2005; 2009a; b; 2010a; b). ChE activity was assessed by the colorimetric method of Ellman et al. (1961), and protein concentrations were obtained

using the bicinchoninic acid (BCA) protein assay (Pierce Biotechnology, Inc., Rockford, IL).

### **Seizure prevention experiment**

Approximately 1 week before experimentation guinea pigs were anesthetized with isoflurane and prepared for recording of electroencephalogram (EEG) by implanting cortical stainless steel screw electrodes using previously described procedures (Shih and McDonough 1997; Shih et al. 2003). On the day of study, the animals were placed in recording chambers, and at least 15 min of baseline EEG was recorded. EEGs were recorded using CDE 1902 amplifiers and displayed on a computer running Spike2 software (Cambridge Electronic Design, Ltd., Cambridge, UK). After the 15-min baseline EEG recording, the animals were challenged s.c. with 2 × LD<sub>50</sub> of sarin (84.0 µg/kg), cyclosarin (114.0 µg/kg), or VX (16.0 µg/kg). Previous work with this model (Shih et al. 2003; 2007) shows that a 2 × LD<sub>50</sub> challenge level with these agents under these conditions will result in seizures in all animals unless they are provided additional anti-convulsant treatment. One minute after nerve agent challenge, the animal was treated with atropine sulfate (0.5 mg/kg, i.m.) plus one of the following doses of pro-2-PAM (13.5, 18.0, 24.0, 32.0, or 43.0 mg/kg, i.m.). In a second smaller experiment, animals challenged with 2 × LD<sub>50</sub> of VX and treated with atropine sulfate (0.5 mg/kg, i.m.) 1 min after challenge were treated with pro-2-PAM (32.0 mg/kg, i.m.) at seizure onset, to determine if delayed pro-2-PAM treatment was capable of terminating ongoing seizure activity. Animals that received cyclosarin challenge also received the oxime MMB-4 (26.0 mg/kg, i.m.) along with the 1 min post-exposure atropine sulfate and pro-2-PAM treatment, since challenge with this agent proved rapidly (< 10 min) lethal with just pro-2-PAM and atropine treatment. Animals were observed continuously for the first hour following exposure and treatment and periodically thereafter for at least 5 h. Recording of EEG was conducted continuously throughout this time and again for another 30 min at 24 h after nerve agent exposure. Seizure onset was operationally defined as the appearance of ≥ 10 s of rhythmic high amplitude spikes or sharp wave activity in the EEG tracing. Each animal was rated as never developing a seizure or having a brief seizure that rapidly terminated (OFF) or developing continuous seizure activity that never stopped (NOT OFF) based on the overall appearance of the EEG record at the end of the experimental day and during the 24-h observation. Animals that survived 24 h were weighed and underwent behavioral testing (see below). Following behavioral testing, animals were deeply anesthetized with sodium pentobarbital (75.0 mg/kg, i.p.) and euthanized by exsanguination via perfusion through the aorta with saline, followed by 10% phosphate buffered formalin (PBF).

### **Neuropathological assessment**

Following post-fixation in PBF for at least 24 h at 4°C, brains were coronally cut into 3-mm slices. Brain slices at the level of the dorsal hippocampus were paraffin-processed and sectioned at 5–10 µm. Sections between bregma –2.52 and –3.12 mm were stained with Hematoxylin and Eosin and

evaluated by an ultrastructural pathologist who was unaware of the experimental history of a given subject. These coordinates contain brain regions that are known to be susceptible to nerve agent-induced damage (McDonough et al. 2000). In the present study, six brain regions (piriform cortex, basolateral amygdala, hippocampus, laterodorsal thalamus, caudate/putamen, and cerebral cortex) were qualitatively scored on a scale from 0–4: 0 = no damage, 1 = minimal damage (1–10% necrotic neurons), 2 = mild (11–25%), 3 = moderate (26–45%), and 4 = severe (> 45%) (McDonough et al. 1995; 2000; Shih et al. 2003). The magnitude of total brain damage was assessed by summing the neuropathology scores of the six areas.

### **Behavioral function study**

Behavioral function was assessed using a two-way shuttle box avoidance test. This test measures the ability of an animal to associate (learning ability) and respond (motor function) to a cue that signals an impending aversive stimulus. Animals that survived 24 h from the seizure prevention experiment (see above) were placed individually into rectangular shuttlebox avoidance chambers (San Diego Instruments, San Diego, CA) modified with the AIRSTIM option. A black acrylic partition with a 20.3-cm (h) × 8.6-cm (w) rectangular aperture at grid-floor level divided the box into two distinct compartments, each ~ 26 cm wide. Pressurized air pulses, 234.4 kPa (34 psi) at 2298 cc/s, 0.5 s in duration with 0.5 s separating each burst (offset to onset), were delivered from four 24.5-cm long aluminum stimulus delivery tubes with an external diameter of 6.5 mm located 19 mm on center above the grid floor on the front and back wall of each box (two per compartment). The air tubes were perforated with nine 2.5-mm × 0.8-mm holes spaced 2.5 cm apart on center. The location of the subjects was detected via 16 infrared photobeams (eight per compartment) located 13 mm above the grid floor. A 5-W mini-bulb house light was centered in the ceiling of each compartment and a 5-W mini-bulb cue light was centered 10.7 cm above the grid floor on the left and right wall of each shuttlebox (one per compartment). In the two-way shuttlebox active avoidance procedure the house light and cue light served as the conditioned stimulus (CS). Five seconds after onset of the CS, the aversive stimulus (air) was presented. The CS remained on during the aversive stimulus. If the animal shuttled to the non-illuminated compartment, the CS and the aversive stimulus were terminated, and a variable intertrial interval (ITI; 20 s, range 15–25 s) was initiated. If the subject did not cross into the non-illuminated compartment following the 15-s aversive stimulus presentation, all stimuli terminated and the ITI began. During the ITI, the subject was allowed to move freely between the two compartments. Responding on each trial was classified as an avoidance response (i.e. crossing to the opposite side after CS onset but prior to aversive stimulus onset), an escape response (i.e. crossing to the opposite side following aversive stimulus onset but prior to its termination), or a 'no response' (i.e. remaining in the compartment throughout the 15-s aversive stimulus presentation). Prior to the start of session, the subjects were allowed

a 5-min adaptation period during which both chambers were dark. Fifty trials were presented in a session (Clark et al. 2003; Myers et al. 2005).

### **Data analysis**

ChE activity was calculated initially as  $\mu\text{mol}$  substrate hydrolyzed/ml/min for blood samples and as  $\mu\text{mol}$  substrate hydrolyzed/g protein/min for brain and peripheral tissue samples. The enzymatic activities of the treatment groups were then expressed as a percentage of the saline-saline control group. Statistical analysis of enzymatic activities was performed using a one-way ANOVA to compare across treatments. A post-hoc Tukey test was used for multiple comparisons. Body weight loss was compared using *t*-test and incidence of neuropathology was evaluated using the Chi-square test. Total time spent in the aversive stimulus, total number of ITI crossings for the entire session, and number of avoidance responses were calculated for each guinea pig and subjected to a one-way ANOVA with three levels of seizure outcome as the categorical predictor (seizure prevented, seizure terminated, or seizure ongoing at 24 h). Following significant main effects, post-hoc comparisons were accomplished using the Newman-Keuls test. Statistical significance for all tests was defined as  $p \leq 0.05$ .

## **Results**

### **Effects of pro-2-PAM on ChE activity**

At 15 min following nerve agent challenge pro-2-PAM produced differential *in vivo* ChE reactivating potency depending on the OP nerve agent that was used. All doses (12.5, 17.7, 25.0, and 35.3 mg/kg) of pro-2-PAM significantly reactivated sarin-inhibited ChE in all three peripheral tissues (heart, diaphragm, and skeletal muscle) and in WB and RBCs (Table 1). Pro-2-PAM at 12.5 mg/kg was not able to reactive sarin-inhibited AChE in any brain samples. The 17.7 and 25.0 mg/kg doses of pro-2-PAM significantly reactivated sarin-inhibited AChE only in the cortex, while the highest dose (35.3 mg/kg) of pro-2-PAM significantly reactivated sarin-inhibited AChE in every CNS tissue tested except the spinal cord (Table 1). All doses of pro-2-PAM significantly reactivated cyclosarin-inhibited ChE in WB and RBCs (Table 2). Only the highest dose of pro-2-PAM (35.3 mg/kg) significantly and selectively reactivated cyclosarin-inhibited ChE in heart and AChE in spinal cord (Table 2). All doses of pro-2-PAM significantly reactivated VX-inhibited ChE in the WB and RBCs, and in each peripheral tissue, with the exception of 12.5 mg/kg in diaphragm and 17.7 mg/kg in skeletal muscle (Table 3). As the doses increased from 17.7 to 35.3 mg/kg, pro-2-PAM progressively reactivated VX-inhibited AChE in more brain tissues. Pro-2-PAM significantly reactivated VX-inhibited AChE in spinal cord (17.7, 25.0, and 35.3 mg/kg), in cortex and hippocampus (25.0 and 35.3 mg/kg), and in cerebellum (35.3 mg/kg). However, even at the highest dose of pro-2-PAM no significant reactivation of VX-inhibited AChE activity was observed in the brainstem, midbrain, or striatum (Table 3). Thus, pro-2-PAM, even at a dose of 12.5 mg/kg,

**Table 1.** The dose-response effects of pro-2-PAM on sarin-inhibited cholinesterase (ChE) and acetylcholinesterase (AChE) activity in brain regions, peripheral tissues and blood of guinea pigs<sup>1</sup>

|                 | Sarin/Saline | pro-2-PAM (12.5 mg/kg) | pro-2-PAM (17.7 mg/kg) | pro-2-PAM (25.0 mg/kg) | pro-2-PAM (35.3 mg/kg) |
|-----------------|--------------|------------------------|------------------------|------------------------|------------------------|
| RBC             | 9.31±2.06    | 27.18±1.62*            | 29.19±1.84*            | 35.39±1.78*            | 41.12±3.05*            |
| WB              | 10.83±1.57   | 36.08±1.43*            | 38.64±1.19*            | 45.53±2.19*            | 50.13±4.57*            |
| Skeletal muscle | 33.25±3.71   | 54.49±6.47*            | 65.35±4.01*            | 73.49±3.51*            | 94.77±8.07*            |
| Diaphragm       | 23.99±2.21   | 48.87±6.16*            | 57.68±5.49*            | 60.20±6.08*            | 77.42±9.11*            |
| Heart           | 12.99±0.73   | 47.96±2.12*            | 53.87±2.55*            | 56.86±4.43*            | 76.85±11.56*           |
| Spinal cord     | 25.78±4.19   | 19.59±5.78             | 19.54±3.70             | 17.35±3.63             | 33.29±13.84            |
| Striatum        | 15.79±2.20   | 22.67±3.10             | 21.15±2.45             | 18.96±2.22             | 37.50±13.12*           |
| Hippocampus     | 16.87±1.76   | 24.07±3.18             | 23.45±2.69             | 20.37±2.16             | 36.03±9.71*            |
| Cortex          | 7.33±0.70    | 13.35±1.70             | 18.03±1.63*            | 17.08±2.21*            | 33.60±8.17*            |
| Cerebellum      | 8.87±1.04    | 12.46±1.96             | 12.44±1.22             | 12.41±0.95             | 29.93±14.66*           |
| Midbrain        | 11.04±1.12   | 15.11±2.12             | 16.99±1.84             | 15.95±1.58             | 30.23±8.51*            |
| Brainstem       | 11.11±1.40   | 14.48±1.59             | 14.19±1.14             | 12.87±0.91             | 31.86±14.48*           |

<sup>1</sup>Data are expressed as percent of saline/saline control ChE activity with mean ± SEM (sarin/saline n=12, pro-2-PAM [12.5mg/kg] n=7, pro-2-PAM [17.7mg/kg] n=8, pro-2-PAM [25mg/kg] n=8, pro-2-PAM [35.3mg/kg] n=4).

\*Statistically different (p<0.05) from sarin/saline group.

**Table 2.** The dose-response effects of pro-2-PAM on cyclosarin-inhibited cholinesterase (ChE) and acetylcholinesterase (AChE) activity in brain regions, peripheral tissues and blood of guinea pigs<sup>1</sup>

|                 | Cyclosarin/Saline | pro-2-PAM (12.5 mg/kg) | pro-2-PAM (17.7 mg/kg) | pro-2-PAM (25.0 mg/kg) | pro-2-PAM (35.3 mg/kg) |
|-----------------|-------------------|------------------------|------------------------|------------------------|------------------------|
| RBC             | 6.19±1.58         | 10.30±0.73*            | 12.15±1.20*            | 12.62±0.80*            | 15.28±1.95*            |
| WB              | 10.28±1.30        | 16.70±1.23*            | 19.23±1.39*            | 22.84±2.11*            | 19.60±1.80*            |
| Skeletal muscle | 62.93±3.94        | 58.84±6.10             | 54.72±5.09             | 61.21±6.91             | 68.16±7.06             |
| Diaphragm       | 44.77±3.36        | 47.65±3.17             | 50.50±2.56             | 48.37±2.17             | 57.16±4.71             |
| Heart           | 26.65±0.75        | 35.80±2.19             | 38.00±1.41             | 40.89±1.12             | 53.57±8.50*            |
| Spinal cord     | 39.79±2.47        | 36.67±2.52             | 39.76±3.02             | 36.20±3.04             | 54.22±6.02*            |
| Striatum        | 29.91±3.72        | 27.93±3.14             | 23.28±2.94             | 20.83±2.40             | 45.38±8.32             |
| Hippocampus     | 33.35±3.08        | 31.31±4.11             | 30.96±2.85             | 29.13±3.80             | 43.96±8.75             |
| Cortex          | 14.54±4.43        | 12.44±2.13             | 11.66±0.85             | 11.93±1.09             | 24.42±9.02             |
| Cerebellum      | 16.81±1.50        | 17.80±2.55             | 18.53±2.87             | 15.42±1.16             | 34.77±14.88            |
| Midbrain        | 24.19±2.48        | 27.02±2.92             | 23.72±2.50             | 24.45±1.49             | 32.04±6.76             |
| Brainstem       | 26.88±2.27        | 27.18±1.62             | 29.19±1.84             | 35.39±1.78             | 41.12±3.05             |

<sup>1</sup>Data are expressed as percent of saline/saline control ChE activity with mean ± SEM (cyclosarin/saline n=10, pro-2-PAM [12.5mg/kg] n=7, pro-2-PAM [17.7mg/kg] n=8, pro-2-PAM [25.0mg/kg] n=8, pro-2-PAM [35.3mg/kg] n=9).

\*Statistically different (p<0.05) from cyclosarin/saline group.

was able to reactivate blood ChE inhibited by all three nerve agents. However, in peripheral and CNS tissues it reactivated sarin- and VX-inhibited ChE in a dose-related manner and showed minimal capacity to reactivate cyclosarin-inhibited ChE activity.

#### Effects of pro-2-PAM on seizures

Table 4 summarizes the efficacy of pro-2-PAM in preventing seizures in guinea pigs exposed to sarin, cyclosarin, and VX. Following sarin challenge, seizure activity developed rapidly ( $\chi=6.3$  min ± 0.22 min SEM,  $n=25$ ) in all animals. Pro-2-PAM at 24.0 and 32.0 mg/kg, administered 1 min after sarin challenge, spontaneously terminated seizures in one of six and two of six animals (Table 4), respectively, with an average time for seizure control (time from pro-2-PAM injection to spontaneously seizure termination) of 39.7 min (± 1.5 min SEM,  $n=3$ ). All other sarin-challenged animals continued to seize, with the exception of the five animals treated with 43.0 mg/kg pro-2-PAM, all of which rapidly died 9–18 min after sarin challenge and pro-2-PAM treatment. Seizures developed rapidly following cyclosarin

challenge ( $\chi=4.9$  min ± 9.2 min SEM,  $n=13$ ). The combination of MMB-4 and pro-2-PAM (18–32 mg/kg) failed to either prevent or stop seizures elicited by cyclosarin challenge in any animal. Seizures developed in a much more protracted fashion following VX challenge ( $\chi=43.3$  min ± 6.9 min SEM,  $n=11$ ). Two animals exposed to VX and treated with pro-2-PAM (one each at 24.0 and 32.0 mg/kg) 1 min after nerve agent challenge failed to develop seizures. These two animals were considered treatment successes (i.e. seizures blocked). In four animals treated with pro-2-PAM (one at 24.0 mg/kg, three at 32.0 mg/kg) seizures developed, but spontaneously terminated 1.75–4.6 h later. These animals were also considered treatment successes. All other VX-challenged animals continued to seize, with the exception of those animals treated with 43 mg/kg pro-2-PAM. The four animals treated with this dose of pro-2-PAM died 17–25 min following VX challenge without ever developing seizures. Since 32.0 mg/kg pro-2-PAM seemed the most successful dose in treating VX-induced seizures, this dose was tested to see if delaying pro-2-PAM treatment until immediately after seizure onset would also be successful in

**Table 3.** The dose-response effects of pro-2-PAM on VX-inhibited cholinesterase (ChE) activity and acetylcholinesterase (AChE) in brain regions, peripheral tissues and blood of guinea pigs<sup>1</sup>

|                 | VX/Saline  | pro-2-PAM (12.5 mg/kg) | pro-2-PAM (17.7 mg/kg) | pro-2-PAM (25.0 mg/kg) | pro-2-PAM (35.3 mg/kg) |
|-----------------|------------|------------------------|------------------------|------------------------|------------------------|
| RBC             | 15.13±3.10 | 28.88±1.80*            | 32.34±2.16*            | 38.62±1.65*            | 43.17±1.76*            |
| WB              | 26.97±3.98 | 36.19±2.69*            | 41.17±2.77*            | 39.58±2.99*            | 47.74±2.42*            |
| Skeletal muscle | 37.92±1.71 | 53.23±5.17*            | 50.39±2.58             | 53.90±4.24*            | 61.13±2.62*            |
| Diaphragm       | 34.66±2.52 | 45.66±3.20             | 46.23±2.70*            | 52.13±1.54*            | 58.25±3.41*            |
| Heart           | 26.81±1.55 | 41.35±2.52*            | 52.96±2.74*            | 57.05±2.28*            | 65.64±3.23*            |
| Spinal cord     | 35.48±4.00 | 43.37±2.19             | 48.67±2.23*            | 51.87±2.30*            | 53.74±4.05*            |
| Striatum        | 43.83±6.06 | 47.04±4.11             | 46.97±2.85             | 52.90±2.14             | 52.99±5.55             |
| Hippocampus     | 16.04±1.02 | 20.63±1.49             | 21.86±1.03             | 25.63±1.48*            | 26.18±2.28*            |
| Cortex          | 12.96±0.63 | 17.86±0.66             | 19.11±1.21             | 22.36±0.97*            | 22.80±3.23*            |
| Cerebellum      | 20.86±1.92 | 20.74±1.53             | 22.05±1.28             | 28.14±2.43             | 29.07±2.54*            |
| Midbrain        | 26.03±2.81 | 26.14±1.46             | 27.29±1.91             | 30.19±2.15             | 33.46±2.89             |
| Brainstem       | 31.20±2.36 | 33.14±2.63             | 34.10±1.48             | 38.05±2.36             | 39.17±3.37             |

<sup>1</sup>Data are expressed as percent of saline/saline control ChE activity with mean ± SEM (VX/saline n=5, pro-2-PAM [12.5mg/kg] n=7, pro-2-PAM [17.7mg/kg] n=8, pro-2-PAM [25.0mg/kg] n=8, pro-2-PAM [35.3mg/kg] n=7).

\* Statistically different ( $p < 0.05$ ) from VX/saline group.

**Table 4.** Efficacy of pro-2-PAM to prevent/terminate seizures in guinea pigs exposed to sarin, cyclosarin, or VX.

| pro-2-PAM Dose (mg/kg) <sup>1</sup> | Sarin              |                  | Cyclosarin         |          | VX                 |                  |
|-------------------------------------|--------------------|------------------|--------------------|----------|--------------------|------------------|
|                                     | Seizure Terminated | Survival         | Seizure Terminated | Survival | Seizure Terminated | Survival         |
| 13.5                                | 0/4                | 2/4              | NT                 | NT       | NT                 | NT               |
| 18.0                                | 0/4                | 1/4              | 0/1                | 0/1      | NT                 | NT               |
| 24.0                                | 1/6                | 2/6              | 0/6                | 0/6      | 2/6 <sup>2</sup>   | 2/6              |
| 32.0                                | 2/6                | 4/6              | 0/6                | 0/6      | 4/6 <sup>2</sup>   | 5/6              |
| 43.0                                | 0/5 <sup>3</sup>   | 0/5 <sup>3</sup> | NT                 | NT       | 0/4 <sup>3</sup>   | 0/4 <sup>3</sup> |

<sup>1</sup>Administered 1 min after agent.

<sup>2</sup>One animal in each of these treatment groups never developed seizure.

<sup>3</sup>All animals died in less than 25 min after pro-2-PAM treatment.

NT=Not Tested.

controlling seizure activity. Pro-2-PAM (32.0 mg/kg) failed to terminate VX-induced seizures when administered at the onset of seizure activity ( $n=4$ ). Across all three nerve agents, animals in which seizures were either prevented or terminated lost significantly ( $t=3.99$ ,  $df=14$ ,  $p<0.001$ ) less body weight ( $\chi=-23.0$  g,  $\pm 4.0$  SEM) in the 24h following exposure than animals that continued to seize ( $\chi=-55.1$  g,  $\pm 6.3$  SEM).

#### Behavioral assessment (shuttle box performance)

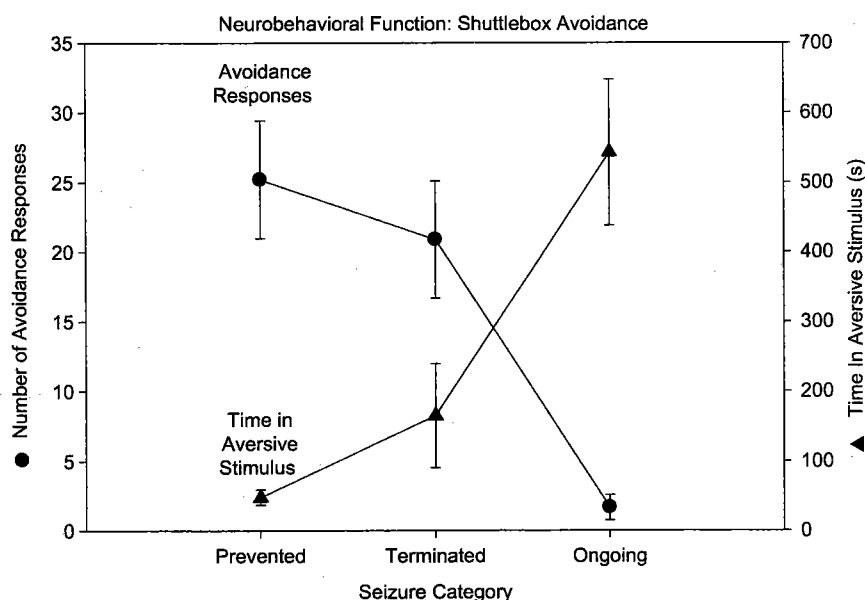
Seizure outcome was predictive of avoidance acquisition and performance (Figure 1). The performances of the seizure-prevented and seizure-terminated groups were comparable and significantly better than that of the seizure-ongoing group for every measure. Specifically, behavioral output was low for the seizure-ongoing group, and this was reflected in significantly fewer avoidance responses ( $F_{2,18}=8.60$ ,  $p<0.003$ ) and ITI crossings ( $F_{2,18}=4.43$ ,  $p<0.03$ ), and significantly greater time spent in the aversive stimulus compared to the two other groups ( $F_{2,18}=8.55$ ,  $p<0.003$ ). Thus, the seizure-ongoing group exhibited profound deficits in avoidance acquisition and performance, likely due to motor impairment. Comparisons between the seizure-prevented and seizure-terminated groups revealed trends toward greater avoidance responding and less time spent in the aversive stimulus for the seizure-prevented group, but these mean differences failed to reach levels of statistical significance.

#### Effects of pro-2-PAM on histopathology

Non-seizure control brains showed a typical neuronal morphology with a pale nucleus and centrally located nucleolus in representative brain regions (CA1 hippocampus (Figure 2a), basolateral amygdala (Figure 2e), piriform cortex (Figure 2i) and striatum (Figure 2m)). Brains from animals that seized but had their seizures spontaneously terminated displayed no necrotic neurons and vacuolar degeneration (Figures 2b, f, j, and n). However, they exhibited altered neuronal morphology characterized by shrunken neurons with ill-defined nuclei and nucleoli. Animals that continued to seize after pro-2-PAM treatment displayed substantial neuropathology marked by severe vacuolar degeneration and abundant eosinophilic necrotic neurons (Figures 2c, d, g, h, k, l, o, and p).

#### Discussion

The results of this study show that pro-2-PAM provided significant in vivo reactivation of both peripheral ChE and central AChE in sarin- and, to a lesser extent, in VX-exposed guinea pigs, but it provided limited peripheral and no central ChE reactivation in cyclosarin-exposed animals. The results of the seizure study parallel these ChE reactivation findings, with dose-dependent protection against sarin- or VX-induced seizures, but not those induced by cyclosarin. Generally, these findings are in good agreement with most of



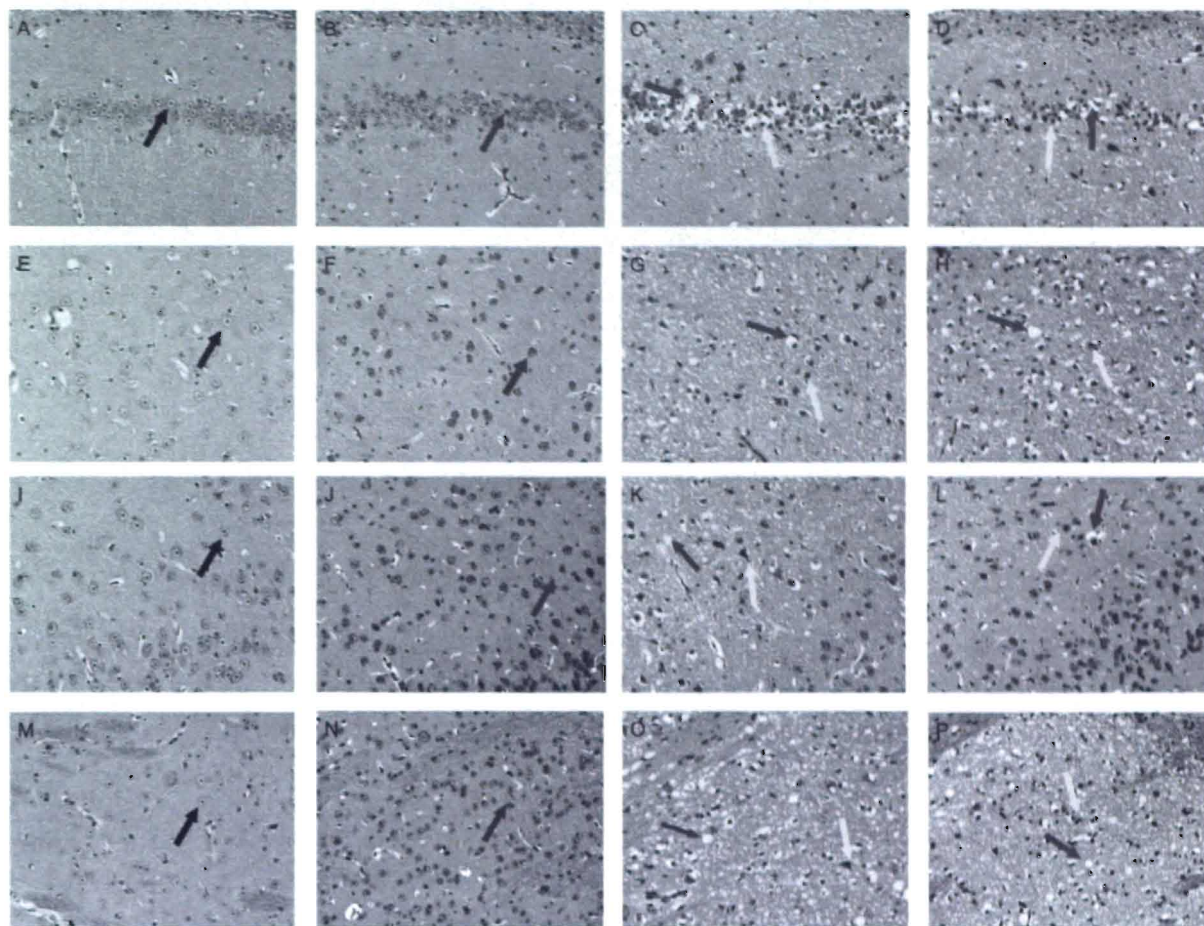
**Figure 1.** Guinea pigs that had ongoing seizures emitted significantly fewer avoidance responses and spent significantly more time in the aversive stimulus than those whose seizures were prevented or terminated. Two measures of shuttlebox avoidance performance in 24-h survivors are shown as a function of seizure category (seizure prevented,  $n=5$ ; seizure terminated,  $n=10$ ; or seizure ongoing,  $n=6$ ). The number of avoidance responses is indicated by circles and corresponds to the left axis. The total time spent in the aversive stimulus (in seconds) is indicated by triangles and corresponds to the right axis. Error bars represent the SEM.

the published literature for OP nerve agents (Clement 1979; Talbot et al. 1986).

In vitro, sarin-inhibited AChE is most readily reactivated by 2-PAM, then VX-inhibited AChE and finally cyclosarin-inhibited AChE (Worek et al. 2004), exactly paralleling the in vivo reactivation findings of this and other studies (Shih et al. 2009a). The fact that peripheral tissues, particularly blood, were readily reactivated even at the lower doses of pro-2-PAM is not surprising, considering that pro-2-PAM undergoes rapid conversion to 2-PAM in the blood ( $t_{1/2} = 1.04$  min; Shek et al. 1976a). Thus, high concentrations of 2-PAM would be readily available in blood and peripheral tissues for reactivation. However, only the higher doses of pro-2-PAM were capable of reactivating AChE inhibited in selected brain regions following sarin or VX, but not cyclosarin exposure. This is probably due to several factors including limited brain penetration of pro-2-PAM under the conditions of this study and the different chemical structures of the nerve agents, which lead to the different relative reactivating potencies against AChE inhibited by the three different nerve agents. Only a small amount of pro-2-PAM actually enters the brain to be converted to 2-PAM (Shek et al. 1976b) and intravascular administration is superior to intramuscular administration of pro-2-PAM in producing a high concentration of active 2-PAM in the brain (Kenley et al. 1982). Once in the brain, sarin- followed by VX-inhibited AChE would be more susceptible to reactivation by 2-PAM than cyclosarin-inhibited enzyme (Worek et al. 2004; Shih et al. 2009a).

The seizure protection results of the second experiment are in concordance with the brain AChE reactivation data. Pro-2-PAM was capable of preventing sarin- and VX-induced

seizures (33% and 66% at the 32 mg/kg dose, respectively), but not those produced by cyclosarin. The inability of pro-2-PAM to reactivate cyclosarin-inhibited brain and muscle (diaphragm) ChE was reflected in the rapid lethal effects of the  $2 \times LD_{50}$  challenge of cyclosarin. Even when MMB-4 was administered, an oxime that is capable of reactivating cyclosarin-inhibited ChE in blood and peripheral tissues (Shih et al. 2009a) and which prevented these rapid lethal effects, pro-2-PAM was not capable of preventing cyclosarin-induced seizures, most likely due to its inability to reactivate brain AChE. Animals in which seizure activity was prevented or rapidly terminated by pro-2-PAM following sarin- or VX-challenge lost significantly less body weight, were behaviorally less debilitated, and were protected from seizure-induced neuropathology, in contrast to the animals that developed continuous seizure activity. However, animals that continued to seize exhibited extensive neuronal injury in all brain regions examined. These results are in good agreement with research that shows that prevention or control of nerve agent-induced seizure activity with anti-convulsant drugs or other protective treatments results in less weight loss, preservation of normal behavioral function, and partial or total protection against brain damage (Harris et al. 1984; Philippens et al. 1992; McDonough et al. 1999; 2000; Shih et al. 2003; 2007). It is interesting that delaying administration of pro-2-PAM until after seizure onset was unsuccessful in controlling seizure activity. This is unlike the seizure protective effects that have been recently observed (Shih et al. 2009b; 2010a; b) with monoisonitrosamine (MINA), a tertiary monooxime capable of penetrating the brain and reactivating sarin-, cyclosarin-, or VX-inhibited AChE (Askew 1956; 1957). MINA was highly effective in preventing seizure occurrence when administered



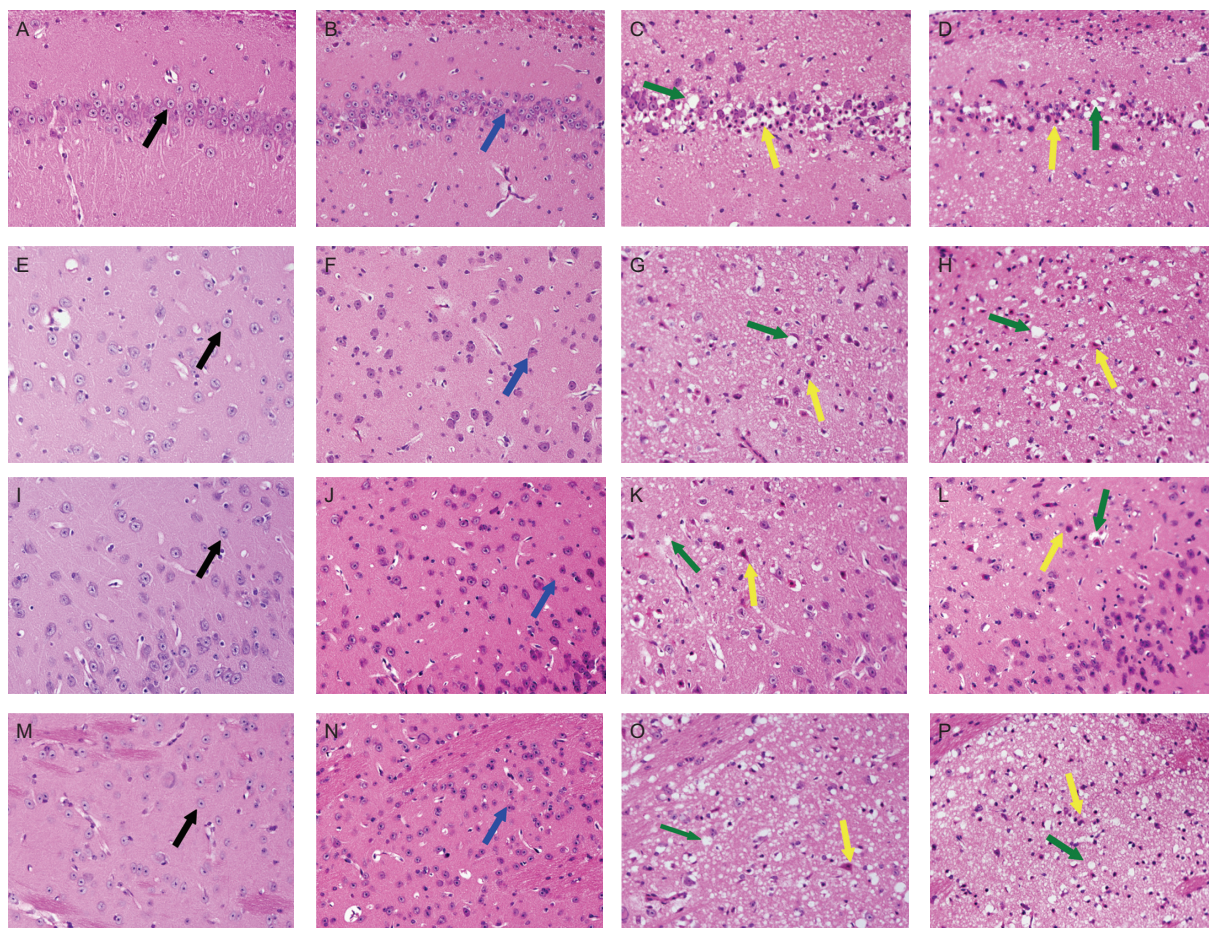
**Figure 2.** Representative micrographs of neuronal damage in CA1 hippocampus (A-D), basolateral (BL) amygdalae (E-H), piriform cortex (I-L) and striatum (M-P). Control brain sections showed normal neuronal morphology with a well-defined nucleus and centrally located nucleolus (black arrows) in (A) CA1, (E) BL amygdala, (I) piriform cortex, and (M) striatum. Although animals that received pro-2-PAM at 1 min after VX or sarin and that had their seizures spontaneously terminated exhibited no overt neuropathology, morphologically altered neurons with shrinking cell bodies were evident (blue arrows) in (B), (F), (J), and (N). Sarin- and VX-exposed animals that continued to seize after pro-2-PAM treatment displayed severe brain damage characterized by necrotic neurons (yellow arrows) and prominent vacuolar degeneration (green arrows) in CA1 (C, sarin; D, VX), BL amygdala (G, sarin; H, VX), piriform cortex (K, sarin; L, VX) and striatum (O, sarin; P, VX). Magnification 200 $\times$ .

1 min, as pro-2-PAM was in this current study, after nerve agent challenge. Moreover, treatment with MINA could be delayed for up to 40 min after seizure activity was initiated by any of these three nerve agents, and seizures could be stopped in a similar fashion as if an anti-convulsant drug had been given (McDonough et al. 2009; Shih et al. 2010a).

At doses  $\geq 43.0$  mg/kg, pro-2-PAM shows signs of toxicity in guinea pigs. This oxime was tested up to doses of 50.0 mg/kg in the reactivation study, but all animals died less than 25 min after administration (data not shown). In the seizure study, animals treated with the 43.0 mg/kg dose died in a similar time-frame, while animals treated with lower doses survived for much longer times. These observations are in agreement with Clement (1979), who found that at doses of 40.0–80.0 mg/kg of pro-2-PAM alone, the animals (guinea pigs) became lethargic, developed tremors, and then died of respiratory failure. Signs similar to these were observed in this experiment. The mechanism of this toxicity is unknown. Since doses of 25–35 mg/kg pro-2-PAM provided the greatest brain reactivation and the most robust protection against seizures,

a relatively narrow safety margin is indicated between therapeutic and toxic doses. Based on the results of this study, pro-2-PAM may not be an effective treatment for chemical warfare nerve agent poisoning, but it may have therapeutic value in DFP poisoning, as reported by other studies (Shek et al. 1976b; Rump et al. 1978; Clement 1979; Kenley et al. 1982; DeMar et al. 2010).

In summary, pro-2-PAM produced at best only a moderate amount of reactivation of sarin- and VX-inhibited AChE in the CNS, prevented seizures and neuropathology, and alleviated behavior deficits in some animals challenged with sarin and VX. Pro-2-PAM produced 9–25% reactivation of cyclosarin-inhibited ChE in blood, heart, and spinal cord, but no reactivation in brain or muscle tissues, and was unable to control cyclosarin-induced seizures. Although pro-2-PAM showed some capacity to reactivate AChE in the CNS and prevent seizures following selected nerve agent intoxication, its moderate reactivation rate and toxicity would appear to limit its usefulness as a ChE reactivator for OP nerve agent intoxication.



**Figure 2.** Representative micrographs of neuronal damage in CA1 hippocampus (A-D), basolateral (BL) amygdalae (E-H), piriform cortex (I-L) and striatum (M-P). Control brain sections showed normal neuronal morphology with a well-defined nucleus and centrally located nucleolus (black arrows) in (A) CA1, (E) BL amygdala, (I) piriform cortex, and (M) striatum. Although animals that received pro-2-PAM at 1 min after VX or sarin and that had their seizures spontaneously terminated exhibited no overt neuropathology, morphologically altered neurons with shrinking cell bodies were evident (blue arrows) in (B), (F), (J), and (N). Sarin- and VX-exposed animals that continued to seize after pro-2-PAM treatment displayed severe brain damage characterized by necrotic neurons (yellow arrows) and prominent vacuolar degeneration (green arrows) in CA1 (C, sarin; d, VX), BL amygdala (G, sarin; H, - VX), piriform cortex (K, sarin; L, VX) and striatum (O, sarin; P, VX). Magnification 200 $\times$ .

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Research was conducted in compliance with the Animal Welfare Act of 1966 (P.L. 89-544), as amended and other Federal statutes and regulations relating to animals and experiments involving animals and adhered to the principles stated in the *Guide for the Care and Use of Laboratory Animals* (National Research Council 1996). The research environment and protocols for animal experimentation were approved by the Institutional Animal Care and Use Committee (IACUC) of the US Army Medical Research Institute of Chemical Defense. The facility where this research was conducted is fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC). The opinions or assertions contained herein are the private views of the authors, and are not to be construed as reflecting the views of the Department of the Army or the Department of Defense.

## Declaration of interest

The authors declare no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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